

# THE ISOLATION OF FRIEND VIRUS PROTEIN BY ZONAL CENTRIFUGATION

An abstract of a Thesis by  
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The problem. Isolation of Friend virus protein in an increased yield for immunological purposes by zonal centrifugation was the purpose of this experiment.

Procedure. Sucrose gradient zonal centrifugation was used to isolate Friend virus polysomes. The concentration of the polysomes and the removal of the sucrose was accomplished by ultrafiltration. Puromycin was used to release the nascent viral protein from the polysomes isolated, and the nascent protein was separated from the polysomes by centrifugation and then concentrated using ultrafiltration.

Findings. 1480 mg of protein was isolated from 30 Friend virus infected mice spleens. This amount was significantly more than the amount expected. Aggregates of soluble protein are believed to have sedimented along with polysomes and these proteins may contaminate the Friend virus protein isolated. Only partial release of the nascent protein was obtained in this study.

Conclusion. The use of sucrose as a separating medium does not separate large aggregates of soluble protein from the polysomes during zonal centrifugation. The use of puromycin in large amounts to release a usable quantity of nascent protein may make this method impractical due to high cost.

Recommendations. Cesium chloride should be used in place of sucrose in zonal centrifugation in an effort to separate the aggregates of soluble protein from the polysomes. An attempt to increase the release of nascent protein with puromycin should be made. The isolated protein should be inoculated into BALB/c mice to ascertain the effectiveness of the viral protein as an immunizing agent and the possible deleterious effect of the contaminating soluble protein.

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ZONAL CENTRIFUGATION

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Presented to  
The School of Graduate Studies  
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by  
Micheal Joye Gilbreath  
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## INTRODUCTION AND REVIEW OF THE LITERATURE

Studies of possible immune protection against viral-induced cancer require the isolation of a suitable antigen. Pure viral capsid protein could serve as an antigen to induce protective antibody production against viral-induced leukemia in mice. This study is concerned with the isolation of such protein in suitable quantities. In the past whole virus particles which had been formalin treated or heat inactivated were used to induce immune protection against oncogenic viruses (Chan, Chirigos and Hook, 1969; de The and O'Connor, 1966). A yield of protein free of virus RNA would prevent the transformation of host cells by the viral RNA, encountered in previous methods used.

The ease of induction of Friend virus leukemia and the high concentration of virus in the plasma (Friend, 1957), and spleen (Chan, et al., 1969) renders the disease an excellent object for study. Several viruses have been described which are capable of producing leukemia in mice (Furth and Metcalf, 1958). However, Friend virus is of unusual interest, since it induces leukemia in a high percentage of adult mice within a few weeks (Metcalf, Furth and Buffett, 1959). In Friend disease there is early, intense proliferation of primitive splenic cells, some of which mature to erythroblasts (Siegler and Rich, 1964). Pathogenesis of Friend virus leukemia has been studied in detail (Metcalf, et al., 1959),

and has been shown that it is possible to transmit the disease to adult Swiss mice or DBA/2 mice (Friend, 1957) through various routes of virus administration: intraperitoneally, subcutaneously, intracerebrally, and intramuscularly. The random-bred Swiss mice and the relatively scarce DBA/2 strain used in earlier studies have been replaced by the more common, inbred BALB/c strain. Serial passage of Friend virus-infected splenic filtrates through inbred BALB/c mice induces in these mice a disease with most of the parameters originally described by Friend in random-bred Swiss mice (Fieldsteel, Dawson and Bostick, 1963).

The early stages of the disease are confined to the red pulp of the spleen. Pronounced splenomegaly results, and it has been concluded that the disease is virus-induced leukemia characterized by independent proliferation of erythroblast and reticulum cells.

Various techniques have been used in the past to concentrate and purify viruses and subcellular particles. Guskey and Wolff (1972) showed a method for concentrating and purifying viral capsid protein from whole virus by combining ultrafiltration and isopycnic centrifugation. The use of trypsin in ultrafiltration of the viral protein facilitated the removal of protein contaminants, but a major disadvantage in this method is that viral capsid proteins are susceptible to the action of 0.25% trypsin. Ultrafiltration at this stage has the added disadvantage in that certain

substances, including serum, are retained by the membrane and must be removed by other means prior to the virus concentration.

Another possible source of capsid protein involves the isolation of coat protein from the viral pre-assembly soluble protein present in host cytoplasm. However, it must be remembered that not all proteins coded by viral RNA are capsid protein. Enzymes concerned with viral replication are synthesized early in the cycle. Summer, Maizel and Darnell (1965) showed at least 12 new proteins, including four viral capsid proteins, in cells infected with poliovirus. These may be enzymes required for viral synthesis, or regulatory ones that interfere with or block certain cellular functions that are not to the advantage of viral replication. The presence of these viral capsid protein has been demonstrated to shut off "early" virus protein synthesis (Sugiyama and Nakada, 1967). But the inclusion in the host cytoplasm of the numerous host cell proteins would make isolation of the capsid protein difficult. Present also in the cytoplasm is host and viral RNA, and any physical or chemical separation technique to eliminate the nucleic acid could have an adverse affect on the proteins.

A third possibility involves the isolation of polysomes containing nascent viral protein. An advantage in this method is the ease of isolating polysomes, but it would be necessary to isolate a large amount of available polysomes



in order to obtain an usable quantity of viral protein from them.

Previous workers have had excellent success in concentration and purification of a variety of murine, feline and avian ribonucleic acid tumor viruses propagated in cell cultures (Toplin and Sottong, 1972; Burger and Noronha, 1970; Larkin and Dutcher, 1970; Oroszlan, Johns and Rich, 1965). Little has been done to further isolate workable quantities of viral proteins by any of these methods. The production of a vaccine against Friend virus disease would depend greatly on the ability to isolate pure viral protein in large amounts. Since ribosomes and polyribosomes can be separated by ultracentrifugation (Cline and Ryel, 1971; Talal, 1966), and since protein synthesis is known to take place on the polyribosomes, the first step in the isolation of Friend virus capsid protein could be the isolation of the viral polysomes. Host cell protein synthesis diminishes following infection by viruses, with viral specific protein synthesis showing a corresponding increase as the infective cycle proceeds, and an increase in polysomes which at this time contain viral protein (Dalgarno, Cox and Martin, 1967; Penman, Becker and Darnell, 1964). Removal of the nascent protein from the polysomes will ensure that almost exclusively viral protein is being isolated free from extraneous material.

For many years the efficient separation of particles

based on either differences in sedimentation rate or differences in density has been obtained in the small swinging-bucket type rotor. However, this method is limited by the quantity of the sample material which can be processed in a single tube. The availability of rotors with large cylindrical cavities has significantly increased the scale of zone-type centrifuge separation. Cylindrical rotors now in use have capacities 25 to 1000 times that of typical swinging-bucket rotor tubes.

It seems plausible that the isolation of Friend virus capsid protein can effectively be done by combining zonal centrifugation and ultrafiltration. Zonal centrifugation combines most of the functions of the analytical centrifuge with the functions of the preparative centrifuge by the collection of analytical data while obtaining preparative quantities of product. An important function of the zonal method is the increased yield of fractionated particles.

By using the zonal centrifugation technique, a particle having a sedimentation value can, in theory, be isolated in a pure state. In a typical sucrose density gradient analysis of rat spleen polyribosomes various peaks corresponding to aggregates of monomers, dimers, trimers and tetramers, could be seen, judged by their relative position in the gradient and the known position of monomers (Talal, 1966). A variation of this method could be adapted to the isolation of viral polysomes from the spleen of mice infected with Friend disease.

Polyribosome preparations from mouse spleen, in contrast to rat spleen, often show a major monomer peak, with the amount of polyribosomes varying inversely with aggregate size, suggestive of polyribosome breakdown caused by ribonuclease (Talal, 1966). This degradation of mouse spleen polyribosomes can be minimized by the addition of an amount of rat liver supernatant fluid. This supernatant fluid separated during high speed centrifugation from rat liver cells is effective in inhibiting the degradative action of spleen endonuclease on the polyribosomes of spleen cells (Northup, Hammond and La Via, 1967; Blobel and Potter, 1966a).

As much as 1.0 to 1.4 mg of ribosomal protein per gram of spleen can be obtained from normal rat spleen. The ratio of free to bound ribosomes is approximately 1.6:1.0 (Talal, 1966). There is reason to expect that a similar, if not a greater yield, of ribosomal protein could be obtained from tumor cells actively synthesizing viral protein. However, it has been reported that the ratio of free to bound ribosome is smaller in tumor cells (Talal and Kaltreider, 1968). To ensure the greatest release of membrane bound ribosomes a suitable detergent, such as Triton X-100, can be added during homogenization (Blobel and Potter, 1967).

A density gradient analysis of the isolated centrifugal fraction can reveal the distribution of ribosomal aggregates. Measurement of optical density of the effluent volume at 280 nm and the simultaneous fraction collection can

simplify the pooling of polyribosomes of corresponding size range. By suitable calculations, the sedimentation of the particles in any centrifugal fraction may be determined, and this value can serve to identify the desired polyribosomes.

Viral protein can be released from polyribosomes by treatment in vitro with puromycin. This inhibitor of protein synthesis is thought to cause the release of nascent polypeptide chains by attaching to the growing end in place of the amino acid-transfer RNA complex (Wettstein and Noll, 1965; Nathans, 1964; Allen and Zamecnik, 1962; Yarmolinsky and De La Haba, 1959). Viral proteins can be separated from the puromycin and further concentrated at this phase by ultrafiltration, thus presenting what is believed to be a relatively pure suspension of Friend virus capsid protein, ideally suited for immunological studies.

#### MATERIALS AND METHODS

A  $10^{-3}$  dose of Friend virus was prepared from a pool of Friend virus which had an  $LD_{50}$  of  $10^4$  per 0.2 ml (Helm, 1970). Then 0.2 ml of this  $10^{-3}$  dose was injected intraperitoneally into 5 week old, male BALB/c mice obtained from Simonsen Laboratory, Gilroy, California. The mice were kept for a period of 4 weeks before being killed by cerebral dislocation and their spleens were excised and weighed. Only spleens weighing 2.0 grams or greater were considered

infected and used. Spleens were washed in cold TKM buffer (0.05M Trishydroxymethylaminomethane, pH 7.5, 0.025 M KCL, 0.005M  $MgCl_2$ ) (Blobel and Potter, 1966b) and two spleens were minced and placed with 35 ml of ice cold 0.25M sucrose-TKM and 5 ml of rat liver supernatant fluid in a Dounce homogenizer which had been previously cooled in an ice bath. Homogenization involved 10 strokes with a loose Teflon pestle (0.001 inch clearance).

The spleen homogenate was then centrifuged in a Lourdes Model A-2 angle head centrifuge at  $0^\circ$ , for 10 minutes, at 13,000 x g to remove large subcellular particles and mitochondria. The mitochondrial free supernatant fluid was again centrifuged at 29,000 x g for 1 hour to prepare a microsomal pellet.

In preparing the pellet for zonal centrifugation, ten tubes with microsomal pellets obtained from 20 mice spleens were homogenized in 10 ml of a 5% sucrose mixture made with 8.9 ml of 5.6% sucrose, 0.1 ml of Triton X-100, and 1.0 ml of rat liver supernatant fluid. The suspension was chilled in an ice bath before being placed in the zonal head.

The zonal gradient preparation followed the procedure outlined by Cline and Ryel (1971). Solutions for the discontinuous sucrose gradient were made from a 66% stock solution. However, Table VI of Cline and Ryel was found to be erroneous, and the Viscosity and Density Tables of Sober (1968) were used.

In most cases the following gradient was used in the zonal A1-14 head: 10% sucrose - 40 ml, 17% - 52 ml, 21% - 62 ml, 24% - 71 ml, 27% - 81 ml, 30% - 94 ml, 32% - 86 ml, 34% - 60 ml, 35% - 60 ml. The gradient components were pumped in using a Sigmamotor, Model T-8 pump at a rate of 20 ml per minute. Tygon plastic tubing (1/8 I.D., 1/4 O.D.) was used and the loading temperature of the zonal head was +2°C, at a loading speed of 2500 RPM.

A 10 - 15 ml sample of the microsomal pellets was injected using a syringe attached to the loading tube, which was cooled in an ice bath to protect the sample. The injection rate was 5 ml per minute. An overlay of 65 - 70 ml of 0.5% sucrose-TKM was pumped in at a rate of 5 ml per minute following the sample injection. The zonal head was prepared for high-speed running and the rotor was accelerated from the loading speed of 2500 RPM to 30,000 RPM and run for 240 min at 0°C.

At the completion of the run, the head was prepared for unloading and the fractions were unloaded at a rate of 12 ml per minute. The outlet tube was adapted to Tygon tubing (1/16 I.D., 3/16 O.D.) and the fractions were analyzed using a Gilson UV monitor at  $280 \pm 10$  nm, and collected at 70 sec intervals in a Buchler refrigerated fraction collector. Each fraction contained 11 - 13 ml and were frozen for future use.

Sedimentation coefficients of the particles in each

fraction were calculated from the following data: volume in each fraction tube and consequent calculation of radius of rotation in the rotor (Cline and Ryel, 1971), refractive index and corresponding viscosity and density of each fraction (Sober, 1968). Calculation of sedimentation coefficients were made using the following formula (Bishop, 1970):

$$S_{20,w,i} = \frac{\rho_p - \rho_{20w}}{\omega^2 t \eta_{20w}} \sum_{f_0}^{f_i} \frac{\eta_{Tmf_i} (R_i - R_{i-1})}{\rho_p - \rho_{Tmf_i} \frac{(R_i + R_{i-1})}{2}}$$

$S_{20,w,i}$  is the sedimentation rate that a particle (that is found in fraction  $i$  of a certain zonal centrifugation) would have if it were sedimenting in water at 20°C.  $\rho_p$  is the assumed density of the particle.  $\rho_{20w}$  is the density of water at 20°C (0.9982 g/ml).  $\omega^2$  is the square of the angular velocity.  $\omega$  is radius/sec.  $t$  is the elapsed time of centrifugation at that velocity.  $\eta_{20w}$  is the viscosity of water at 20°C (1.002 in centipoises).

In the summation, the  $\sum$  value for particles in fraction  $i$  ( $f_i$ ) is the run constant on the left times the sum of the expression on the right, summing the calculations for all fractions from the starting position of the sample up to fraction  $i$ .

$\eta_{Tmf_i}$  is the viscosity of the medium at the temperature of the run for the medium in fraction  $i$ .  $\rho_{Tmf_i}$  is the

density of the medium in fraction  $\underline{i}$ .  $R_i$  is the radius of rotation at the outer edge of fraction  $\underline{i}$  when in the centrifuge (Cline and Ryel, 1971).  $R_{i-1}$  is the similar radius for the previous fraction.  $R_o$  is the radius at the outside of the sample zone.  $R_i - R_{i-1}$  is the distance a particle moves going through  $\underline{i}$ .  $\frac{R_i + R_{i-1}}{2}$  is the average radius of rotation for fraction  $\underline{i}$ .

For a few summations this is laborious, but not prohibitive. Doing this for 50 samples would be very time consuming, and a simple computer program makes it much more convenient. For use in this program three values constant for each run are read into the computer.  $M_o$  = radius at the outside of the sample zone.  $A$  = the assumed density of the particle ( $\rho_p = 1.41$ ) (Anderson, Harris, Barber, Rankin and Candler, 1966).  $B = \frac{(\rho_p - 0.9982) \times 2}{\omega^2 t \times 1.002}$

values for each fraction are also read into the computer.  $P_i$  = density,  $N_i$  = viscosity,  $M_i$  = radius at the outside, for fraction  $\underline{i}$ .

The read-out from the computer is:  $P_i$ ,  $M_i$ ,  $N_i$ ,  $\underline{s}$  increment for the fraction,  $\underline{s}_i$  summed up to and including fraction  $\underline{i}$ ,  $\underline{i}$  the sample number.

After several runs the fractions were removed, thawed and pooled according to the polysome peaks shown on the recorder read-out sheets, and by general range of sedimentation



coefficient values. The fractions were then filtered in an Amicon ultrafiltration apparatus, using a membrane UM20E with a pore size of approximately 15,000-25,000 mw, to remove the sucrose and to concentrate the polysomes. The polysomes were retained by the filter and were then washed three times with TKM buffer and concentrated by filtration to a final volume of 50 ml.

Puromycin was added to the concentrated polysome fractions according to a method adapted from Blobel and Sabatini (1971). Additional runs following this method were made to test the effectiveness of  $10^{-4}M$  and  $10^{-5}M$  concentration of puromycin in releasing the nascent protein from the polysomes. Rat liver from previously hepatectomized rats was used in the preliminary experiments involving  $H^3$  and varying concentrations of puromycin and reaction times of 15 min and 30 min.

A  $10^{-3}M$  concentration of puromycin was added to the pooled fractions that had been concentrated to an absorbance of approximately 50. The mixture was allowed to react for at least 30 min and centrifuged at  $99,000 \times g$ , in a Beckman 50.1 angle head, for 1 hr, to separate the protein-puromycin complex from the polysomes. The polysome pellet was saved for further use.

The suspension containing the protein-puromycin complex was filtered using the Amicon ultrafiltration apparatus and UM05 membrane, pore size 500 mw, to remove the puromycin

from the protein fraction. The protein fraction was concentrated to approximately a 10 ml volume and washed twice with 5 ml of TKM buffer to aide in removing any remaining puromycin and then filtered to a final volume of 10 ml.

The puromycin filtrate was then used when homogenizing the polysome pellets obtained in the previous step, and allowed to react again, in an effort to release additional protein from the polysomes. Both volumes of protein were kept separate and the yield of nascent protein from both was determined by UV absorbance and then frozen.

#### DATA AND RESULTS

Liver from previously hepatectomized rats was used initially to establish a suitable gradient and to find the position of the monomer peak. A typical sucrose density gradient analysis of rat liver polyribosomes is shown in Figure 1.

The first peak to arise is found in the fraction range which corresponds to soluble protein and lipoprotein sedimentation values. The second major peak corresponds to the monomer peak as judged by its relative position in the gradient and the known position of monomers. It was found to have a  $S_{20w}$  value between 83 and 111, a little higher than 79, as reported for other mammalian single ribosomes (Talal, 1966). The third and final peak was found to have

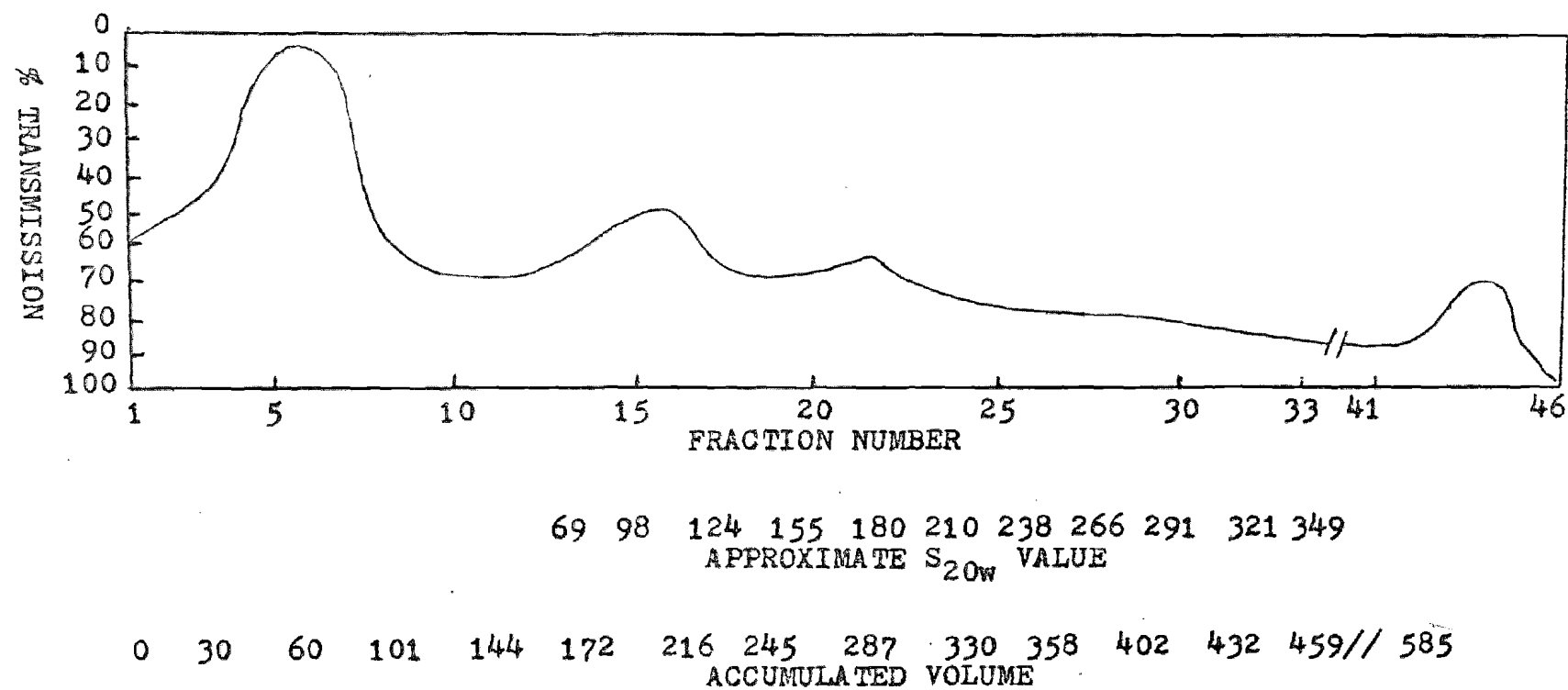


Figure 1. Typical sucrose density gradient analysis of rat liver polyribosomes.

a  $S_{20w}$  value of between 170 and 200, indicative of an aggregate of dimers. Difficulty was found in obtaining any significant peaks corresponding to aggregates larger than dimers when using rat liver.

In subsequent runs rat spleens were used to confirm the appearance and position of the peaks found in the rat liver sample, and to see if any new peaks would appear when using rat spleen as sample material. The results of the runs using rat spleen confirmed the results of Talal (1966), in that only two major peaks were demonstrated; the soluble protein fraction and a monomer peak. The monomer peak appeared larger than the monomer peak seen using the rat liver preparation.

In the first zonal runs in which Friend virus infected spleens were used, no preparative concentrating of the sample was done. In an early attempt, a gummy suspension resulted from the initial homogenization when four infected spleens were added to the 40 ml of buffer-TKM and rat liver supernatant fluid. No explanation for this was determined. In order to prevent this from happening in later runs, homogenization was restricted to using two Friend virus infected spleens per 40 ml of the TKM-rat liver supernatant fluid. The spleen homogenate was then centrifuged as described above to prepare the microsomal pellets. This procedure also served to concentrate the sample material to be used in the zonal runs. 15 to 20 of the microsomal pellets were then

combined and prepared as described for the sample preparation.

A typical sucrose density analysis of Friend virus infected mouse spleen is shown in Figure 2. The first peak represents the soluble fraction, which is well below the  $S_{20w}$  value for single ribosomes. This first peak was found to be somewhat smaller than the soluble fraction found in the rat liver analysis. The second peak, starting around fraction 11, shows no distinct division from the end of the soluble fraction. This second peak is probably representative of where the monomer begins. Some of the sucrose components used in making up the zonal gradient were varied in an effort to sharpen up the individual peaks and to separate them better. However, no significant improvement was obtained.

There is a noticeable increase in the total amount of polysomes present in the broad shouldering peak between fractions 11 and 28. Included in this peak are particles in the  $S_{20w}$  range of 120 and 350 which indicates that the bulk of the polysomes appear as dimers, trimers and tetramers. Fractions in this range were collected and frozen.

Several polysome fractions were measured by UV absorbance to determine the average optical density of the fractions. The average was approximately 8.0 to 8.5 O.D.<sub>260</sub>. The polysomes were concentrated by ultrafiltration for two reasons. The first, to remove the sucrose; the second, to concentrate the polysomes to a 50 O.D.<sub>260</sub> value. An Amicon

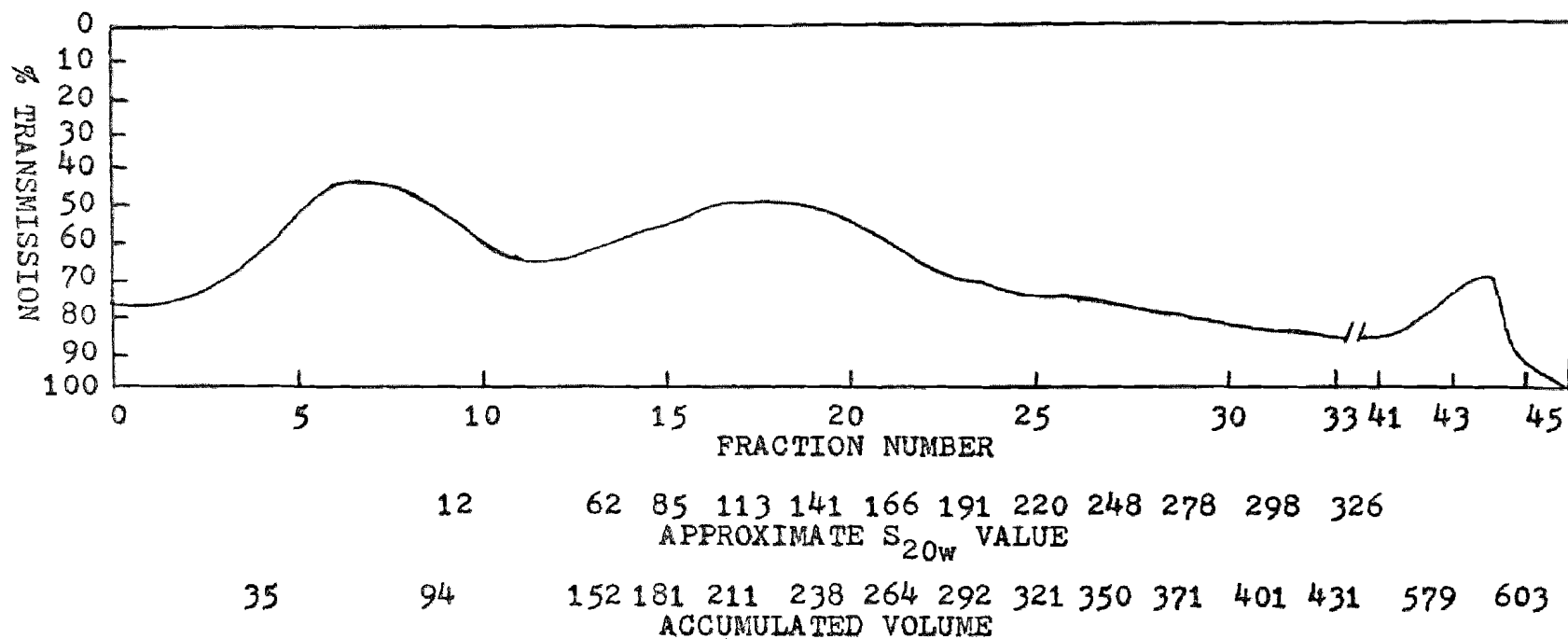


Figure 2. Typical sucrose density analysis of Friend virus infected mouse spleen.

membrane, UM20E, at 60-70 psi, was used for this purpose. Polysomes from the entire range were concentrated into one batch. Eighty-three tubes containing 15 ml/tube, equaled approximately 1250 ml which had an O.D.<sub>260</sub> value of 8.0. 510 ml of this suspension was concentrated to 45 ml by ultra-filtration and washed several times with TKM buffer to obtain a suspension having an O.D.<sub>260</sub> value of 50.

The method of Blobel and Sabatini (1971) was used to test the effectiveness of a less concentrate solution of puromycin. It was postulated that a  $10^{-4}\text{M}$  or  $10^{-5}\text{M}$  concentration could be as effective as a  $10^{-4}\text{M}$  solution if allowed to react with the polysomes for a longer time. Previously hepatectomized rat liver was used in this experiment.

Figure 3 represents the average release of nascent protein for seven sample disc. A wide variation of values for individual samples was found. For example, the  $10^{-3}\text{M}$  concentration of puromycin that was allowed to react for 30 min, showed a range of values from 34 - 50% release. This compared to the value of 20 - 36% release for the polysomes in which no puromycin was used. However, in each individual test, the corresponding increase of nascent protein was around 14% higher for the  $10^{-3}\text{M}$  concentration of puromycin than when no puromycin was used. The  $10^{-4}\text{M}$  and the  $10^{-5}\text{M}$  concentration of puromycin showed a 5 or 15% lower release respectively. The longer reaction times with puromycin did show some small increase in per cent of protein released.

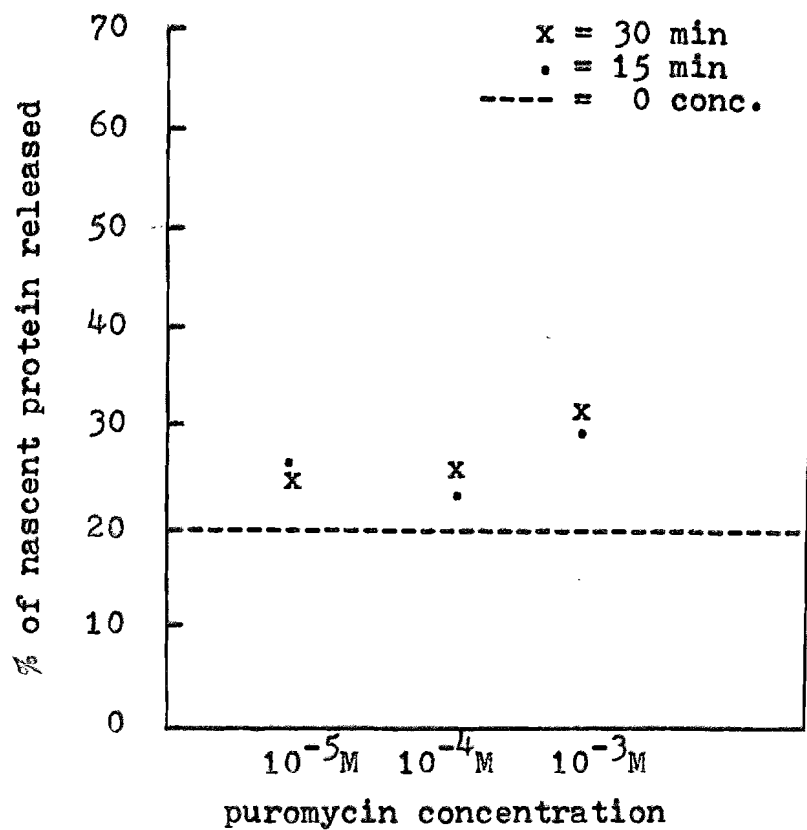


Figure 3. Average release of nascent protein from polysomes at varying concentrations of puromycin and varying times as compared to the release of nascent protein when no puromycin is used.



Only rarely did the  $10^{-4}\text{M}$  concentration which reacted for 30 min show a value higher than the  $10^{-3}\text{M}$  concentration that reacted for only 15 min.

A  $10^{-3}\text{M}$  concentration of puromycin that is allowed to react for at least 30 min was determined to be most effective from Figure 3. Thirty-four 15 ml tubes having an absorbance of 8.0 were concentrated by ultrafiltration to a volume of 45 ml and an absorbance of 50. Puromycin was added following the method of Blobel and Sabatini (1971). The mixture was allowed to react for at least 30 min. The mixture was centrifuged in a Beckman 50.1 angle head for 1 hr at 135,000 x g to separate the polysomes from the protein-puromycin complex. The protein suspension was removed for ultrafiltration and the polysome pellets were retained for use later. The absorbance of the supernatant fluid was 25.

The protein-puromycin complex was filtered using an Amicon filtrating apparatus with a UM05 membrane to remove the puromycin and to concentrate the protein. When the volume had been reduced to 20 ml, the protein was washed twice with 20 ml of TKM buffer to remove any residual puromycin and then concentrated by filtration to a final volume of 20 ml. The absorbance of the protein was 71 absorbance units at 260 nm and 82 absorbance units at 280 nm. Using Packer's formula (Packer, 1967) the total amount of protein isolated was found to be 1480 mg.

## DISCUSSION

The amount of protein isolated is greater than the expected yield. Assuming that there is approximately 1.4 mg of ribosomal protein/g of spleen tissue (Talal, 1968), 2.5 mg of ribosome/g of spleen tissue can be isolated (Tso, Bonner and Dintzin, 1958). Ribosomes fall in the range  $4.0 \times 10^6$ mw (Tashiro and Yphantis, 1965). The range for nascent protein is assumed to be  $4 \times 10^4$ mw. Therefore, 1% of the ribosomal weight is protein. From the 2.5 mg of ribosome/g of spleen tissue, 0.025 mg protein/g of spleen tissue can be expected. Since 30 infected spleens weighing an average of 2.7 grams were used, approximately 2.0 mg of nascent protein could be expected to be isolated, compared to the 1480 mg isolated.

The large yield of protein may have resulted from aggregates of soluble protein sedimenting in the range associated with the range for polysomes during the zonal centrifugation. The extended period of freezing and the additional manipulation during ultrafiltration and washing may have broken these proteins into smaller particles that did not sediment with the polysomes during centrifugation to separate the polysomes from the protein-puromycin complex.

The apparent sedimentation of these soluble proteins may be responsible for the inability to obtain any good resolution between the monomers, dimers and larger polysome aggregates. If this is the case, the soluble protein could

be removed after zonal centrifugation by using cesium chloride as a separating medium in place of sucrose. The lighter soluble protein would separate based on the difference in density between the proteins and the polysomes. However, the use of cesium chloride is more expensive.

It may not be necessary to separate these soluble proteins from the nascent protein. The bulk of these proteins may be pre-viral, regulatory, or synthesizing proteins needed for the synthesis of viral protein and may be important for the immunity against Friend virus disease in mice. This can be tested by injecting some of the bulk isolated protein into mice and then challenging the mice with Friend virus. An additional group of mice inoculated with only the nascent protein could indicate what effects are caused by the protein.

The enhancement of the release of the nascent protein by puromycin must be considered further in this method. The results in this experiment were not as high for the release of nascent protein by puromycin as the results presented by Blobel and Sabatini (1971). However, the reference point of Blobel and Sabatini for 0% release may actually be the average value for release of nascent protein without puromycin. It is possible that the protein-puromycin complex sedimented in part, with the polysome fraction during centrifugation, however, no proof of this exists. Since only 40% of the nascent protein is released by a  $10^{-3}$ M concentration of puromycin it may be necessary to increase the

concentration of puromycin. The additional expense of puromycin may rule out the feasibility of this method as an inexpensive method for isolating a usable quantity of nascent viral protein. A second approach which might show success is the re-use of the puromycin separated from the nascent protein that was first isolated.

The use of cesium chloride as a separating medium and the development of an improved method for releasing the nascent protein with an inexpensive amount of puromycin appears to be an important requisite for the application of this method to the isolation of Friend virus protein with increased yields. Ultimately the significance of this method will depend on the immunological data obtained from the protein isolated by this method.

#### CONCLUSION

A preparation designed to contain only nascent viral protein contained much more protein than could have been present as nascent protein. The polysome fractions obtained by zonal centrifugation with sucrose as a separating medium may contain contaminating protein aggregates which sediment in the range of polysomes. It may be necessary to use cesium chloride as a gradient to remove the protein complexes for the Friend virus protein to be of immunological importance.

A  $10^{-3}M$  concentration of puromycin releases

approximately 40% of the nascent protein. A higher yield of nascent protein would render this method more practical, however, the additional cost of puromycin may prohibit the use of this method as an inexpensive method to obtain an increase quantity of viral protein.

The use of the viral protein for immunological testing depends on the effects caused by the contaminating soluble protein and the difficulty encountered in removing the unwanted protein. The significance of the use of this method for obtaining Friend virus protein depends ultimately on the development of immunity to Friend virus disease by BALB/c mice immunized with the isolated protein.

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